THE VELOCITY OF FIXATION OF COMPLEMENT WITH BACTERIAL ANTIGENS

STUDIES ON COMPLEMENT FIXATION. VI

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In a recent paper from this laboratory,[1] it was shown that the velocity of fixation of complement with protein antigens and specific serums was not markedly affected by temperatures ranging from about 8 to 37 C. In these experiments, 2 vegetable proteins, edestin and phaseolin, of high purity were employed with immune rabbit serums. The temperatures of fixation were icebox (8-12 C.), room (18-22 C.) and water bath (37.5 C.) and the degree of fixation was measured at intervals extending from 5 minutes to 6 hours. These studies further indicated that the velocity of fixation of complement was directly proportional to the number of complement-fixing substances in the immune serum; that the greater part of complement was "fixed" during the first hour and that fixation was practically completed at the end of 4 hours.

More recently the velocity of fixation of complement in the Wassermann test was studied.[2] Six different extract antigens were employed. It was found that water bath, room, and icebox temperatures gave practically the same degree of fixation with all antigens studied except with one prepared according to Noguchi. The last antigen gave a somewhat higher degree of fixation at water bath than at icebox temperature. A fixation period of 4 hours at icebox temperature, however, was found to approach complete fixation of complement with all antigens, including the Noguchi.

The interest which has developed in recent years in complement fixation with bacterial antigens suggested the studies presented in this paper. It was desired to find to what extent different temperatures would affect the velocity of fixation of complement with bacterial antigens, and also the time and temperature of optimum fixation with different antigen-antibody complexes.

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1 Kahn, R. L.: Jour. Exper. Med., 1921, 34, p. 217.

2 Kahn, R. L., and Olin, R. M., Jr.: Jour. Infect. Dis., 1921, 29, p. 630.

EXPERIMENTS

Antigens.—Six different antigens were employed. These were prepared from B. typhosus, B. paratyphosus A, B. paratyphosus B, B. abortus, B. pullorum and B. mallei, respectively. The organisms were obtained from stock cultures of this laboratory. These were first grown in broth for about 18 hours and then transferred on meat infusion agar of suitable P_H. The agar flasks were incubated from 24 to 48 hours, depending on the amount of growth. After incubation, the flasks were placed in the icebox over night. This, we believe, rendered it easier to wash off the organisms without breaking off particles of agar. To each flask were added 5 to 7 c c of normal salt solution and the growth washed off by means of a sterile cotton swab. The bacterial suspension was then placed in a graduated test tube and centrifuged at high speed. The

TABLE 1

VELOCITY OF FIXATION OF COMPLEMENT AT ICEBOX, ROOM, AND WATER-BATH TEMPERATURES

Fixation		Antityphoid Serum (C c)									No. of Positive Signs Denoting Degree of Fixation
Period, Min.	Temperature	0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	
0	.....	1*	1	1	1	—	—	—	—	—	4
5	Water bath.	3	3	3	1	1	1	—	—	—	12
	Room.....	3	3	3	1	1	1	—	—	—	12
	Icebox.....	4	4	1	1	1	1	—	—	—	12
15	Water bath.	4	4	4	4	2	1	—	—	—	19
	Room.....	4	4	4	4	2	2	—	—	—	20
	Icebox.....	4	4	4	4	1	1	—	—	—	18
30	Water bath.	4	4	4	4	4	4	1	—	—	25
	Room.....	4	4	4	4	4	4	1	—	—	25
	Icebox.....	4	4	4	4	4	1	—	—	—	21
60	Water bath.	4	4	4	4	4	4	2	1	—	27
	Room.....	4	4	4	4	4	4	3	1	—	28
	Icebox.....	4	4	4	4	4	4	1	—	—	25
120	Water bath.	4	4	4	4	4	4	4	3	1	32
	Room.....	4	4	4	4	4	4	4	4	—	32
	Icebox.....	4	4	4	4	4	4	4	2	—	30
180	Icebox.....	4	4	4	4	4	4	4	4	—	32
240	Icebox.....	4	4	4	4	4	4	4	4	1	33
300	Icebox.....	4	4	4	4	4	4	4	4	1	33
300	Icebox.....	4	4	4	4	4	4	4	4	1	33

* 4 = ++++, 3 = +++, 2 = ++, 1 = +, and — = negative.

supernatant salt solution was pipetted off as completely as possible and 15 c c ether per 1 c c of packed organisms added. The tube was shaken vigorously and ether extraction permitted for 6 hours. At the end of this period, the ether was filtered off and the organisms were dried on the filter paper until no ether odor could be detected. The organisms were then resuspended in salt solution and titrated for their complement-binding, anticomplementary and hemolytic properties in the usual manner.

The employment of ether in connection with the preparation of bacterial antigens is highly desirable, according to one of us (Johnson). Ether extrac-

tion appears to reduce markedly the anticomplementary properties of these antigens. Bacterial antigens prepared by previous extraction with ether have been kept in the icebox in this laboratory for over a year without acquiring anticomplementary properties.

Immune Serums.—With the exception of the case of B. abortus in which bovine serum was employed, the specific serums were obtained from rabbits. These animals were immunized with the various organisms in the usual manner and the serums employed after they were found to contain a sufficient number of antibodies.

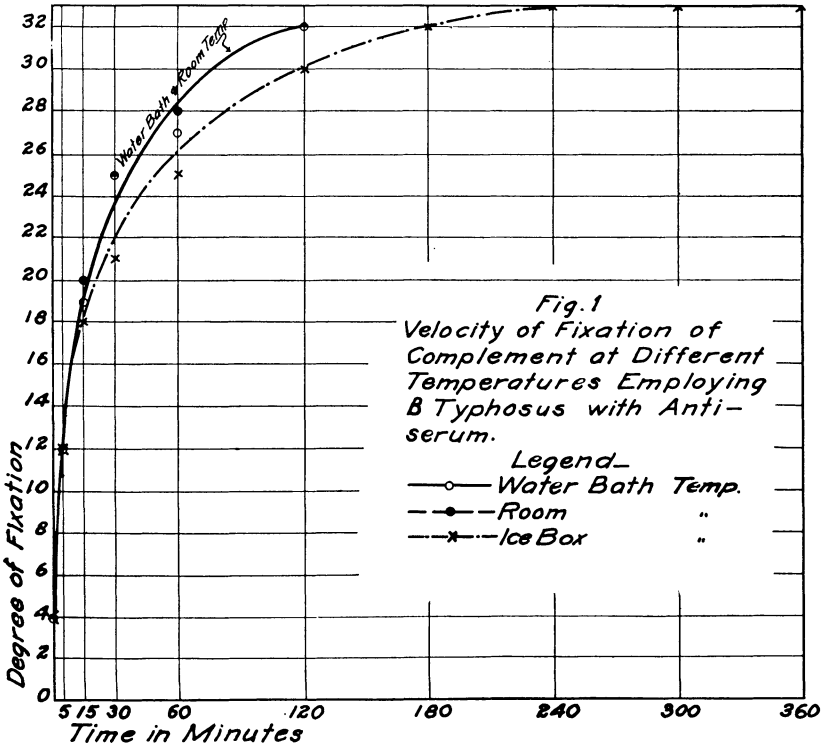
Complement-Fixation Tests.—These were carried out with a sheep cell system. All ingredients entering into the test were used in 0.1 c c quantities except the immune serums, which were graded from 0.01 to 0.0001 c c. Two units of amboceptor, 2 units of complement and from 2 to 3 units of the various antigens were employed. After a given fixation period, 0.1 c c of the standard 5% sheep cell suspension and 0.1 c c amboceptor (2 units) were added and incubated in the water bath (37.5 C.) for about 15 minutes, when the serum and antigen controls would be completely hemolyzed. All final readings were made after keeping the tubes in the icebox over night. The usual plus sign system was employed in recording the results.

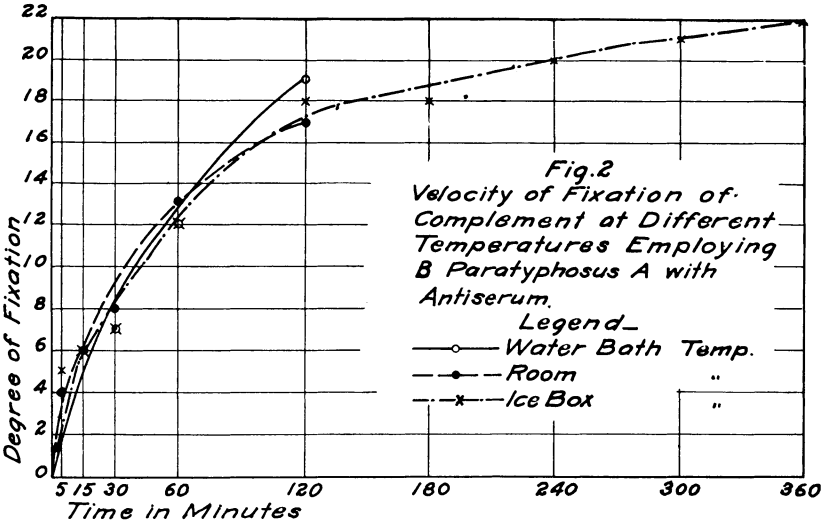
Table 1 gives an outline of the first experiment carried out with a typhoid antigen and antityphoid rabbit serum. The fixation periods were 0, 5, 15 and 30 minutes and 1, 2, 3, 4, 5 and 6 hours. It will be noted that the fixation periods at water-bath and room temperatures were not extended beyond 2 hours. This, because of marked deterioration of complement which takes place after prolonged exposure at these temperatures.

This table indicates that the velocity of fixation of complement with B. typhosus and antityphoid serum is somewhat greater at water-bath and room temperatures than icebox temperature. The difference, however, is comparatively small. Similar results have been obtained with B. paratyphosus A and B, B. abortus and B. mallei with their respective serums. B. pullorum gave considerably more fixation at water-bath than at icebox temperature. Figures 1 to 6 illustrate the findings with the various bacterial antigens. Although these represent single experiments, they may be considered as being representative of each antigen-antibody complex, the experiments in each case having been repeated no less than 3 times and in some cases as many as 10 times, with similar results.

DISCUSSION

A glance at the charts would indicate that a period of fixation of 1 hour in the water bath, generally employed in complement-fixation tests with bacterial antigens, does not represent a sufficient interval for maximum fixation in many cases. It is true that when employing


Water Bath
Room Temp
Degree of Fixation
Time in Minutes
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
5 15 30 60 120 180 240 300 360
Fig. 1
Velocity of Fixation of Complement at Different Temperatures Employing B Typhosus with Anti-serum.
Legend_
Water Bath Temp.
Room "
Ice Box "


Degree of Fixation
Time in Minutes
0
2
4
6
8
10
12
14
16
18
20
22
5 15 30 60 120 180 240 300 360
Fig. 2
Velocity of Fixation of Complement at Different Temperatures Employing B Paratyphosus A with Antiserum.
Legend_
Water Bath Temp.
Room "
Ice Box "

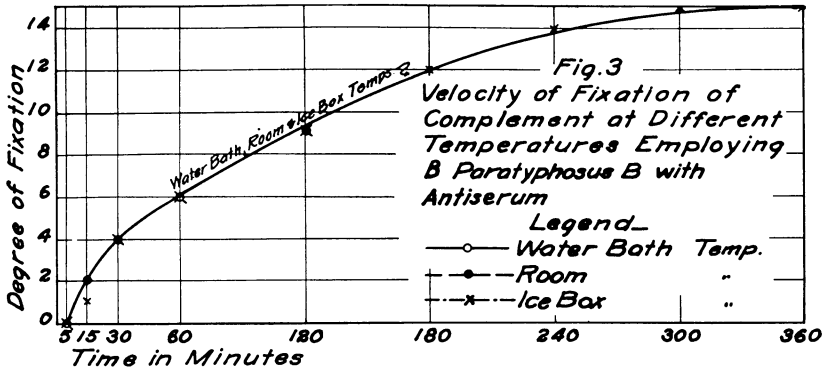


Fig. 3
Velocity of Fixation of Complement at Different Temperatures Employing B Paratyphosus B with Antiserum
Legend—
Water Bath Temp.
Room "
Ice Box "

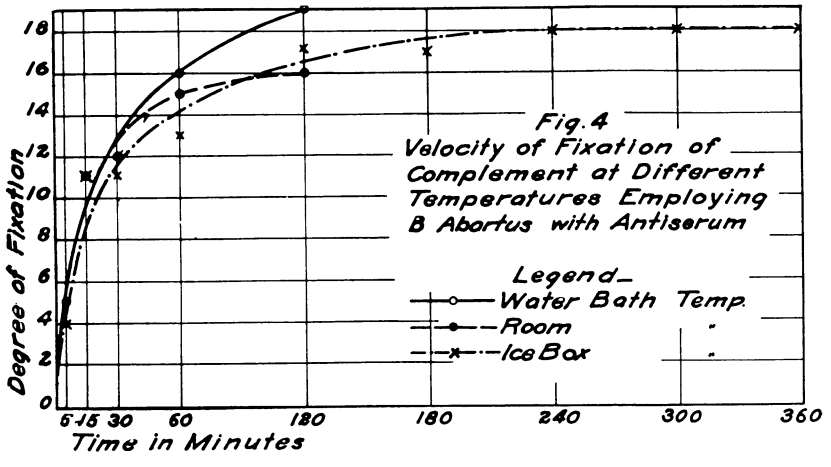


Fig. 4
Velocity of Fixation of Complement at Different Temperatures Employing B Abortus with Antiserum
Legend—
Water Bath Temp.
Room "
Ice Box "

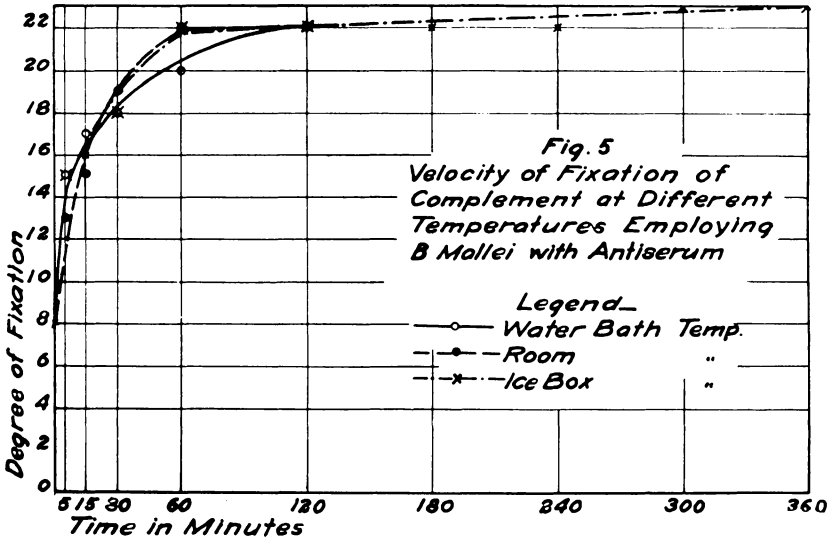


Fig. 5
Velocity of Fixation of Complement at Different Temperatures Employing B Mallei with Antiserum

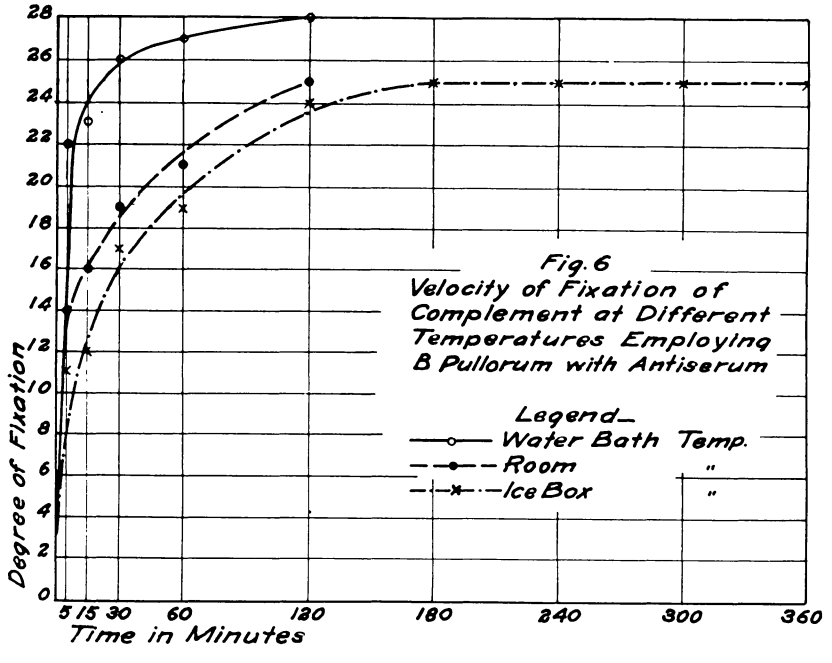


Fig. 6
Velocity of Fixation of Complement at Different Temperatures Employing B Pullorum with Antiserum

strongly positive serums a 1-hour period is quite ample; indeed, even less than 1 hour is sufficient in some instances. In the case of weak serums, however, one will frequently obtain no more than about 50% of fixation during this period. And the detection of weak complement-fixation reactions may, at times, prove to be of greater diagnostic value than the detection of strong reactions. The reason for this lies in the fact that strong complement-fixation reactions are usually accompanied by clinical manifestations of a disease, whereas in the case of weak reactions, the laboratory finding may serve as the only clue in establishing the presence of the disease. A fixation period limited to 1 hour, therefore, is likely to weaken just those reactions which ought to be rendered as sensitive as possible.

That a period limited to 1 hour is insufficient for complete fixation of complement in the Wassermann test, has been shown in a previous paper.[2] It was further shown that a period of 4 hours at ordinary icebox temperature appears to approach maximum fixation of complement in that test. Kolmer,[3] in his recently standarized complement-fixation test for syphilis, requires a fixation period of 18 hours in the icebox. It is not unlikely that the difference between the complement-fixation procedure of Kolmer and that of this laboratory explains in part our different conclusions with regard to the period of fixation. Although we are inclined to think that 18 hours is more than is needed for complete fixation of complement in the Wassermann test, it would appear, both from the studies of Kolmer and our own, that a fixation period of 1 hour is quite insufficient for these tests. And there is no reason to believe that the inter-reaction of complement with syphilitic serum and extract antigen is essentially different from the inter-reaction of this ingredient with specific antigen-antibody complexes.

Furthermore, whenever it is experimentally established that the velocity of fixation of complement with a given antigen-antibody mixture goes on equally as well at water bath and icebox temperatures, the latter temperature is to be preferred. The reason for this is that complement deteriorates at water bath temperature, whereas it is practically preserved in the icebox. It may be of interest, in this connection, to record the following observation made during the course of these fixation experiments. On adding standard amounts of sheep cells and hemolysin to a series of tests after 1-hour fixation periods at different temperatures, it was observed again and again that the serum and antigen controls hemolyzed far more readily in the tests in which fixa-

[3] Jour. of Syphilis, 1922, 6, p. 82.

tion was carried out in the icebox than in those in which fixation was carried out in the water bath. The explanation for this is clear. In the tests in which fixation was carried out in the icebox, the complement was practically as potent as in the beginning of the experiment and hemolysis of the serum and antigen controls took place readily. In the tests which were "fixed" in the water bath, however, the controls hemolyzed slowly because the complement had somewhat deteriorated during fixation. This explains also why slightly stronger reactions are frequently observed after 2 hours' fixation in the water bath compared with the same period in the icebox, as indicated in some of the charts. The stronger reactions are undoubtedly due in a large measure to complement deterioration rather than to specific fixation. It is evident, since the disappearance of complement forms the underlying basis of complement-fixation tests, that every factor which tends to destroy complement is to be eliminated from this test.

Assuming that a 1-hour period of fixation at water bath temperature is not conducive to dependable complement-fixation results with bacterial antigens, the question arises, What should be the underlying basis for correct fixation with these antigens? Judging from our results, it would appear that with 5 out of the 6 organisms studied a fixation period of 4 hours at ordinary icebox temperature approaches complete fixation of complement and may be taken as a reasonably safe procedure. Aside from the fact, however, that our studies are limited to this small number of organisms, it is conceivable that even with these organisms different complement fixation procedures might affect the phenomenon of fixation. We would say, therefore, when undertaking a series of complement-fixation studies with a given bacterial antigen, that the optimum time and temperature of fixation should be determined in that case in the same way as other optimum conditions of the test are determined. This can be accomplished readily by carrying out several experiments as outlined in table 1.

For correct complement-fixation tests, we insist on titrating a number of ingredients, and there is no reason why we should not also determine the proper mode of fixation by special titration. Our present criteria for fixation of complement with bacterial antigens are based largely on empirical data obtained with several strongly positive and negative serums. What is needed is some quantitative measurements of fixation at different temperatures carried out with different dilutions of positive serum and, in our opinion, the outline indicated in table 1 answers the purpose. One may leave out the 0, 5 and 15

minute fixation periods from this velocity experiment. But the other phases of this outline should be adhered to. Three different immune serums tested with a given bacterial antigen as outlined in the table should indicate the optimum mode of fixation with that antigen.

It should not be assumed that the attempt to find the optimum time and temperature of fixation with each bacterial antigen will lead to marked variations in the method of fixation on the part of different workers. On the contrary, judging from our complement-fixation studies with protein, Wassermann, as well as bacterial, antigens, we are inclined to believe that different workers will, in most cases, approach the same method of fixation. We believe also that icebox fixation, in many cases, will be found to be superior to water-bath fixation. The period of fixation, however, will have to be judged in each case by the nonspecific complement-binding properties of the bacterial antigen resulting from prolonged incubation. If, for example, after 4 hours of fixation in the icebox there is a tendency for a given bacterial antigen to be anticomplementary, the fixation period may have to be limited to 2 hours. This fixation period would be found to be more than ample with strong serums and would assure about 80% of fixation with weak serums.

SUMMARY AND CONCLUSIONS

The velocity of fixation of complement at icebox, room and water-bath temperatures was studied with the following 6 antigen-antibody complexes: B. abortus with specific bovine serum, B. typhosus, B. paratyphosus A and B, B. mallei and B. pullorum with specific rabbit serum. The fixation periods in each case were 0, 5, 15 and 30 minutes and 1, 2, 3, 4, 5 and 6 hours.

It was observed that the velocity of fixation of complement is practically the same at the different temperatures studied. This was true with all organisms except B. pullorum. With this organism, fixation at water-bath temperature was more marked than at icebox and room temperatures.

It was further observed that with the exception of B. pullorum, a fixation period of 4 hours at ordinary icebox temperature is superior to a 1-hour period in the water bath. This period of fixation in the water bath was found to be of insufficient length to bring about complete fixation of complement with 5 of the organisms studied. Furthermore, the employment of water bath temperature for fixation of complement should be avoided as far as possible because this temperature hastens the deterioration of complement.

Finally, it was pointed out that the mode of fixation of complement with a given bacterial antigen should be determined by special "titration" in the same way that various other factors of the complement-fixation test are determined by titration. The numerous ways of preparing bacterial antigens combined with the varying complement-fixation procedures employed by different workers, in our opinion, necessitates this step until we learn more of the laws governing the phenomenon of complement fixation. A simple outline for determining the optimum temperature and time of fixation of complement with different bacterial antigen-antibody complexes is indicated.